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Immune response mediated by liposome-associated protein antigens

IV. MODULATION OF ANTIBODY FORMATION BY VESICLE-ENCAPSULATED METHOTREXATE*

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SUMMARY

Large unilamellar reverse-phase evaporation vesicles (REV) were used as a heterobifunctional carrier for a pharmacologically active agent and a protein antigen. Methotrexate (MTX) was encapsulated in the hydrophilic compartment of liposomal REV with or without surface-conjugated bovine serum albumin (BSA) antigen. The administration of MTX encapsulated within BSA-coated vesicles (MTX.REV-BSA) could either enhance or depress the anti-BSA plaque-forming cell (PFC) response in mice. On the other hand, the administration of MTX entrapped in plain vesicles (MTX.REV) produced essentially a suppressive effect on the PFC response stimulated by the simultaneous injection of a separate antigen, e.g. vesicle-conjugated BSA (REV-BSA). This suppression of the antibody response occurred, whether MTX.REV was injected 1 day before, during, or 1 day after the immunization with an antigen. These results indicate that liposome-encapsulation of a very limited drug dose can modify the immunobiological effect of the drug which, when presented in its free form at the same dose, exerts no appreciable action on the engendered immune response. Thus, the modulation of the immune response by a liposome-encapsulated drug appears to be influenced by the drug dose can modify the immunobiological effect of the drug which, when presented in its free form

INTRODUCTION

Liposomes are known to be very effective in promoting antibody formation to protein antigens, and the immunopotentiating properties of liposomal carriers have been reviewed (van Rooijen & van Nieuwmegen, 1982; Alving & Richards, 1983; Shek, 1984). Liposome vesicles can render a protein antigen more immunogenic if the antigen molecules are either entrapped internally (Heath, Edwards & Ryman, 1976; Six, Kramp & Kasel, 1980; Shek & Sabiston, 1982a) or absorbed on the membrane surface (Shek & Sabiston, 1982b; van Rooijen & van Nieuwmegen, 1982). The immunogenicity of externally associated protein antigens can also be observed if the protein molecules are covalently conjugated to the membrane surface (Shek & Heath, 1983; Snyder & Vannier, 1984). Proteins may be coupled to liposomes, which also contain hydrophilic molecules trapped in their aqueous interior (Heath *et al.*, 1983; Matthay *et al.*, 1984). Such a liposomal carrier may allow any vesicle-encapsulated agent to act as a regulator of the immune response engendered by the covalently conjugated antigen molecules. This might especially occur if the interaction of the antigen-coated liposomes with cells resulted in the internalization of the liposomes and the delivery of their aqueous contents to the cells. This approach may be termed antigen-mediated targeting of immunomodulators, and presents at least two interesting possibilities. Firstly, known immunomodulators might be rendered more effective by their delivery to specific cells involved in antigen processing. Secondly, cytotoxic agents which serve only to suppress the immune response by affecting all rapidly dividing cells may be induced to have more specific effects by exclusive delivery to those cells, interacting with the antigen.

In this report, the effect of liposome-entrapped methotrexate (MTX) on the antibody response to bovine serum albumin (BSA), conjugated to the surface of the same or separate vesicles, is described. We find that, whereas encapsulated MTX is normally immunosuppressive, it can stimulate the response to BSA if the antigen is covalently attached to the drug-loaded liposomes.

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Abbreviations: BSA, bovine serum albumin; MTX, methotrexate; MTX.REV, methotrexate-containing vesicles; MTX.REV-BSA, methorexate-containing vesicles with surface-conjugated BSA; PFC, plaque-forming cells; REV, reverse-phase evaporation vesicles; REV-BSA, vesicles with surface-conjugated BSA; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; SRBC, sheep red blood cells.

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MATERIALS AND METHODS

Animals

Male A/J mice, 6–8 weeks old, were supplied by the Jackson Laboratories, Bar Harbor, ME. Animals were kept in plastic cages and were allowed free access to laboratory mouse chow and water.

Chemicals and biological materials

The sources of chemicals and biological materials used in this study were the same as previously described (Shek & Heath, 1983), with the following exception. Methotrexate was obtained from Lederle as the lyophilized sodium salt.

Preparation of reverse-phase evaporation vesicles (REV), encapsulation of methotrexate, and protein conjugation

Reverse-phase evaporation vesicles were prepared and conjugated to BSA as described previously (Shek & Heath, 1983). If MTX was to be encapsulated, the aqueous medium for liposome preparation was a 290 mOsm solution, pH 6·7, containing 50 mM morpholinoethanesulphonic acid, 50 mM morpholinopropanesulphonic acid, 50 mM MTX, and sufficient NaCl to give the required osmolarity. Non-encapsulated drug was removed prior to conjugation by gel chromatography on a 10 × 1·0 cm Sephadex G-75 column equilibrated with the same buffer without MTX. The drug content of the liposomes was determined by extracting an aliquot of the preparation by the method of Bligh & Dyer (1959). The MTX content was measured in the upper aqueous/methanol phase, assuming a molar extinction coefficient for MTX of 7943 at 370 nm.

Vesicle-bound BSA was measured by using the method of Lowry *et al.* (1951) in the presence of sodium deoxycholate. A 200-μl portion of the sample was mixed with 60 μl of 20% (w/v) sodium deoxycholate prior to the addition of 1 ml folin reagent and 100 μl of diluted phenol reagent. For MTX-containing vesicles, the encapsulated drug was found to produce colour in the Lowry test; colour production by MTX was proportional to its concentration. The colour contribution of MTX was controlled for with blanks consisting of the same aliquot of MTX-containing vesicles but without surface-conjugated protein. Consequently, the measured protein content was corrected by a subtraction based upon the MTX content of the liposomes and the determination of an encapsulated MTX solution which contained no protein.

Haemolytic plaque assay

Procedures used for the preparation of spleen cells and for the determination of the plaque-forming cell (PFC) response were performed as previously described in detail (Shek & Sabiston, 1982a).

RESULTS

Effect of liposome-entrapped methotrexate on the PFC response

Antigens conjugated to the surface of MTX-containing vesicles

The BSA-specific PFC response of mice (Fig. 1, Group C) immunized with albumin-conjugated vesicles containing 50 nmol of MTX (MTX.REV-BSA) was significantly enhanced in comparison to control animals (Group A) injected with albumin-conjugated vesicles (REV-BSA) containing no entrapped drug. In contrast, the PFC response was depressed in animals (Group E) injected with twice as much MTX (100 nmol) encapsulated in BSA-coated vesicles. The injection of animals with either 50 nmol (Group B) or 100 nmol (Group D) of free MTX at the time of immunization with REV-BSA did not alter the magnitude of the antibody response compared with that of control animals (Group A).

Because of the opposite effect of the encapsulated MTX on the PFC response observed above, a titration of the drug effect over a larger range, from 12·5 to 100 nmol was performed and the combined results are summarized in Table 1. We have previously shown that the magnitude of the PFC response is essentially the same when as little as 7 μg and as much as 30 μg of vesicle-conjugated BSA are used for immunization (Shek & Heath, 1983). Therefore, within a comparable dose range of conjugated BSA (i.e. 13–30 μg) used for immunization in this drug-effect titration experiment, the observed change in the PFC response shown in Table 1 is attributable to the effect of the encapsulated MTX. It can be seen that maximal immunopoten-

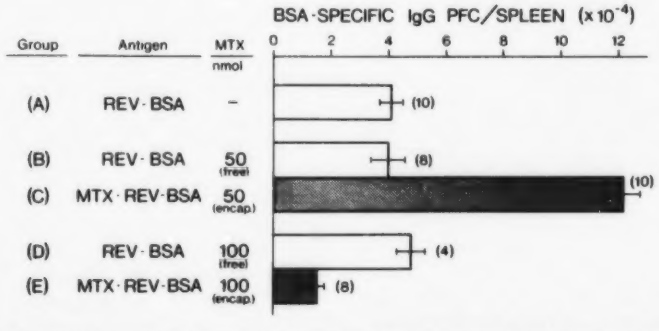

Figure 1. Modulation of the antibody response by liposome-encapsulated methotrexate (MTX). Different groups of mice were given two injections, 3 weeks apart, of about 30 μg BSA conjugated on the surface of liposomal vesicles with or without entrapped MTX. The dose of MTX, in free or encapsulated form, administered to each animal at the time of immunization was either 50 or 100 nmol. The peak BSA-specific PFC response was assayed 4 days after antigenic challenge. Each bar represents the mean response ± SEM of the number of animals indicated in each bracket. *P* values (Student's *t*-test) compare the PFC response among groups: Groups B and C, $P < 0{\cdot}0001$; D and E, $P < 0{\cdot}002$; A, B, and D, not significant. (Abbreviations: REV-BSA, vesicles with surface-conjugated bovine serum albumin; MTX.REV-BSA, methotrexate-containing vesicles with surface-conjugated bovine serum albumin.)

Table 1. Effect of varying doses of MTX encapsulated in BSA-coated vesicles on the anti-BSA PFC response

		Immunizing dose*		BSA-specific antibody response†		
Group	*n*	MTX (nmol)	BSA (μg)	Mean PFC spleen ± SEM	PFC (%)	*P*‡
(A)	15	0	30	39,500 ± 2200	100	—
(B)	8	12·5	13	41,000 ± 2700	104	NS§
(C)	8	25·0	27	67,800 ± 2500	172	< 0·0001
(D)	15	50·0	30	122,800 ± 6500	311	< 0·0001
(E)	8	75·0	21	28,800 ± 2500	73	< 0·005
(F)	8	100·0	28	13,300 ± 1300	34	< 0·0001

* Animals were immunized with two injections of BSA-conjugated liposomes bearing encapsulated MTX. Different liposome preparations, containing 33, 59, and 64 μg of BSA as well as 0·12, 0·10 and 0·06 μmol of MTX, respectively, per μmol of lipid vesicle were used.

† The splenic BSA-specific PFC response was assayed 4 days after antigenic challenge.

‡ *P* values (Student's *t*-test) comparing the mean PFC response with that of Group A.

§ NS, not significant.

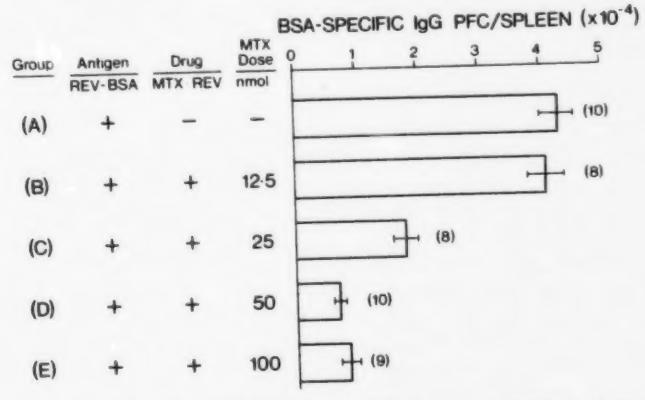

Figure 2. Depression of antibody formation by liposome-encapsulated MTX. Mice were immunized intraperitoneally with 30 μg of REV-BSA twice, 3 weeks apart, and various doses (12·5–100 nmol) of vesicle-entrapped MTX were also administered at the time of each immunization. The peak BSA-specific PFC response was assayed 4 days after antigenic challenge. Each horizontal bar represents the mean response ± SEM of the number of animals indicated in parentheses. *P* values (Student's *t*-test) compare the PFC response among groups: Groups A and B, not significant; A and C, $P < 0{\cdot}0001$; A and D, $P < 0{\cdot}0001$; A and E, $P < 0{\cdot}0001$.

tiation occurred at 50 nmol (Group D), and lower doses of 25 and 12·5 nmol were either less stimulatory (Group C) or ineffective (Group B). Higher doses (75 and 100 nmol) of entrapped MTX exerted a progressively suppressive effect (Groups E and F). The PFC response of control animals injected with a similar dose range of *free* MTX was unaffected (data not shown). Therefore, the immunomodulatory effect of MTX is dependent on its physical entrapment within antigen-bearing liposomes.

Antigens not associated with MTX-containing vesicles

The injection of liposome-entrapped methotrexate (MTX.REV) to animals simultaneously immunized with a separate preparation of liposome-conjugated BSA (REV-BSA) significantly depressed the BSA-specific PFC response (Fig. 2). Although the lowest dose (12·5 nmol) of the entrapped drug was ineffective (Group B), higher doses (25–100 nmol) were clearly suppressive (Groups C–E). This suppression occurred, whether MTX.REV was administered before (Day −1), during (Day 0), or after (Day +1) the injection of the antigen, REV-BSA (Fig. 3).

The antibody response of mice to another antigen, sheep red blood cells (SRBC), was also depressed by the injection of liposome-entrapped methotrexate (Table 2). The progressive depression of the anti-SRBC PFC response correlated with the entrapped drug dose between 25 nmol and 100 nmol (Groups C–E). The injection of as much as 100 nmol of free MTX (Group F) or less (data not shown), however, did not affect the outcome of the anti-SRBC PFC response.

DISCUSSION

Liposome-encapsulated MTX exerts a dose-dependent effect on the immune response to BSA that is conjugated to the surface of

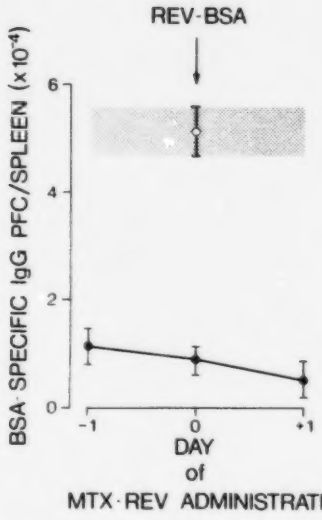

Figure 3. Effect of varying the time of MTX.REV administration on the depression of the PFC response. Groups of mice (eight animals group) were given 100 nmol MTX.REV on the day before, during, and after their immunizations with 30 μg of REV.BSA on Day 0. Control animals received the same dose of antigen, REV.BSA, but no MTX.REV was given. The peak BSA-specific PFC response was assayed 4 days after antigenic challenge. Each point represents the mean response ± SEM of MTX.REV treated animals (●) or of control animals (○).

the liposomes. The injection of 25–50 nmol of MTX encapsulated within BSA-coated liposomes enhanced the PFC response to the BSA antigen. In contrast, 100 nmol of MTX in BSA-coated liposomes depressed the PFC response to the BSA. Free MTX, 12·5–100 nmol, had no effect on the PFC response elicited by BSA conjugated to empty liposomes. Therefore, liposome-encapsulated MTX can mediate a dose-related modulatory effect on the immune response elicited by antigens present on the surface of the same liposomes.

When the immunizing antigen is not presented on the surface of the MTX-loaded liposomes, the results are slightly different. A dose of 25–100 nmol of MTX in unconjugated liposomes significantly depressed the PFC response engendered by BSA conjugated to the surface of a separate liposome population. The suppression of the PFC response occurs, even if the encapsulated MTX is given 1 day before or after the immunization. Encapsulated MTX also suppress the PFC response to SRBC. The administration of 25 and 50 nmol of encapsulated MTX partially suppresses the PFC response to SRBC, while 100 nmol substantially reduces the response.

MTX is an anti-proliferative agent exerting little effect on cells which do not divide. Therefore, the effects of encapsulated MTX on the immune response are likely to involve cells which divide rapidly during the initial phase of the response. Methotrexate has previously been shown to have immunosuppressive effects when 1 mg/kg (50 nmol per 20 g mouse) is administered 2 days after immunization with SRBC (Di Lorenzo *et al.*, 1974). However, MTX has no effects when as much as 4 μmol per mouse are given at the same time as the SRBC antigen (Lin, 1973). Intraperitoneally injected free MTX would diffuse rapidly throughout the body and be rapidly excreted via renal filtration. Intraperitoneally injected liposomes are cleared from the peritoneum via the lymphatics. Therefore, the effects of the drug may be potentiated by retention and slow release within the lymphatic system.

Methotrexate and its derivatives are known to be delivered to cells by carrier-dependent delivery (Heath *et al.*, 1983). Therefore, MTX may exert a much larger effect on cells which take up the liposomes than on other cells. Macrophages have been shown to play an obligatory role in inducing the antibody response to liposome-encapsulated BSA (Shek & Lukovich, 1982). MTX is unlikely to have significant effects on macrophages, because they do not divide. However, the macrophages may carry the liposomes and their contents to the lymph nodes, where the drug will slowly effuse from the macrophages as they degrade the liposomes. This process could explain the immunosuppressive effects of encapsulated MTX. In addition, albumin-coated liposomes may interact directly with proliferating antigen-specific lymphoid cells and may deliver MTX directly to these cells. Such specific delivery could explain the immunostimulatory effects of MTX in BSA-coated liposomes, especially if a subset of lymphocytes, such as the suppressor cells, were involved. Such a mechanism would also be consistent with the

Table 2. Effect of vesicle-encapsulated methotrexate on the anti-SRBC PFC response in A J mice

		Day 0			Day 4	
Group	*n*	Antigen*	Drug†	MTX dose (nmol)	Anti-SRBC IgM PFC spleen‡ (mean ± SEM)	*P*§
(A)	16	SRBC	—	—	104,900 ± 4600	
(B)	8	SRBC	MTX.REV	12·5	103,000 ± 7000	NS
(C)	8	SRBC	MTX.REV	25·0	82,000 ± 5300	< 0·01
(D)	8	SRBC	MTX.REV	50·0	75,300 ± 7200	< 0·002
(E)	8	SRBC	MTX.REV	100·0	25,000 ± 5200	< 0·0001
(F)	9	SRBC	Free MTX	100·0	92,700 ± 6600	NS

* Animals were immunized intraperitoneally (i.p.) with 5×10^6 SRBC.

† Methotrexate (MTX), in its free or vesicle-encapsulated (MTX.REV) form, was administered separately by the i.p. route at the time of immunization.

‡ The peak splenic anti-SRBC PFC response was assayed 4 days after immunization.

§ *P* values (Student's *t*-test) in comparison with the mean PFC response of control animals in Group A.

suppressive effect of 100 nmol of MTX entrapped in BSA-coated liposomes. These BSA-coated liposomes would also be taken up by macrophages. However, at the higher drug dose of 100 nmol, the stimulatory effects due to an interaction with a specific lymphoid cell subset might be overwhelmed by the suppressive effects on the lymphoid system as a whole.

Liposomes provide a very useful means for examining the effects of modulators on the immune system, and ultimately might lead to the formation of potent immunogens. These studies also have great importance for the potential use of liposomes for the delivery of cytotoxic agents to the lymphoid system. It has been suggested that potential complications may arise from the use of antibody-conjugated liposomes for drug delivery, if the liposomes stimulate an immune response to the antibody itself (van Rooijen & van Nieuwmegen, 1982). The results described here demonstrate further complexity which the presence of a cytotoxic drug may introduce. In this context, the stimulation which we have seen at low drug concentrations is a further detraction from the use of liposomes as a drug carrier. However, the suppression of the response which we have seen at higher drug concentrations might be used to overcome the problem, providing that the general effects of immunosuppression are not severe. In summary, our results demonstrate a potentially useful application of drug-containing liposomes in immunomodulation.

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